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Comparison of human and guinea pig acetylcholinesterase sequences and rates of oxime-assisted reactivation

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ABSTRACT

Poisoning via organophosphorus (OP) nerve agents occurs when the OP binds and inhibits the enzyme acetylcholinesterase (AChE). This enzyme is responsible for the metabolism of the neurotransmitter acetylcholine (ACh) which transmits signals between nerves and several key somatic regions. When AChE is inhibited, the signal initiated by ACh is not properly terminated. Excessive levels of ACh result in a cholinergic crisis, and in severe cases can lead to death. Current treatments for OP poisoning involve the administration of atropine, which blocks ACh receptors, and oximes, which reactivate AChE after inhibition. Efforts to improve the safety, efficacy, and broad spectrum utility of these treatments are ongoing and usually require the use of appropriate animal model systems. For OP poisoning, the guinea pig (*Cavia porcellus*) is a commonly used animal model because guinea pigs more closely mirror primate susceptibility to OP poisoning than do other animals such as rats and mice. This is most likely because among rodents and other small mammals, guinea pigs have a very low relative concentration of serum carboxylesterase, an enzyme known to bind OPs *in vitro* and to act as an endogenous bioscavenger *in vivo*. Although guinea pigs historically have been used to test OP poisoning therapies, it has been found recently that guinea pig AChE is substantially more resistant to oxime-mediated reactivation than human AChE. To examine the molecular basis for this difference, we reverse transcribed mRNA encoding guinea pig AChE, amplified the resulting cDNA, and sequenced this product. The nucleotide and deduced amino acid sequences of guinea pig AChE were then compared to the human version. Several amino acid differences were noted, and the predicted locations of these differences were mapped onto a structural model of human AChE. To examine directly how these differences affect oxime-mediated reactivation of AChE after inhibition by OPs, human and guinea pig red blood cell ghosts were prepared and used as sources of AChE, and the relative capacity of several different oximes to reactivate each OP-inhibited AChE were determined. The differences we report between human and guinea pig AChE raise additional concerns about the suitability of the guinea pig as an appropriate small animal model to approximate human responses to OP poisoning and therapies.

1. Introduction

Organophosphorus (OP) nerve agents cause toxic effects by inhibiting acetylcholinesterase (AChE) [1] which acts in nerve junctions to hydrolyze the neurotransmitter acetylcholine (ACh). If it is not hydrolyzed, ACh will increase to levels that cause seizures and other pathophysiological effects which, if not treated, can eventually lead to death. Current treatments for OP nerve agent intoxication include the administration of muscarinic antagonists and AChE-reactivating oximes [1].

Efforts to improve the protective efficacy of treatments for OP nerve agent intoxication are currently being pursued. One of the final steps involved in testing any new treatment involves *in vivo* testing to determine safety and efficacy. The animal model most commonly used to evaluate antidotes for efficacy against OP nerve agent intoxication is the guinea pig. The guinea pig is used because it is a good compromise between non-human primates (which are expensive and often in limited supply) and other small animal models such as mice and rats, where serum carboxylesterase expression results in higher resistance to poisoning by OP nerve agents [2,3].

In addition to the presence of serum carboxylesterase, another shortcoming of the guinea pig as an animal model is that guinea pig AChE has been reported to have different activities when compared to human AChE [4,5]; these differences include both disparity in catalytic efficiency with acetylthiocholine as a substrate, and in the

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capacity of some oximes to mediate reactivation after inhibition by OPs. Complete nucleotide and amino acid sequences of guinea pig AChE have not been published or deposited into DNA or protein databases, preventing a structural analysis of the basis for these differences. We report here the determination of the nucleotide and deduced amino acid sequence of guinea pig AChE, along with initial *in vitro* and *in silico* analyses of this protein.

2. Materials and methods

2.1. Sequencing

Guinea pig cortical brain tissue was freshly isolated and frozen in liquid nitrogen. The brain tissue was disrupted and 30 mg of tissue were homogenized using a Bead Beater (Biospec, Bartlesville, OK). Beads were removed and total RNA was isolated using the RNEasy Kit (Qiagen, Valencia, CA) according to manufacturer's instructions. Primers predicted to be specific for the 3′ end of the guinea pig AChE gene were designed based on a partial sequence available at www.ensembl.org as a shotgun sequence fragment; these fragments are being assembled in anticipation of creating a full guinea pig genome sequence. The primers (GP ACHE Rev1 [5′-GGTCTGGGACTCGTCTGTTA-3′] and polyT [5′-TTTTTTTTTTTTTT-3′]) were used to generate single stranded cDNA. This DNA was amplified by PCR (using primers GP ACHE For1 [5′- CTGCTCTGGCAGCCATGA-3′] and GP ACHE Rev1). Amplified product was gel purified and used as the template for the BigDye® Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). Initial sequence reaction was performed with GP ACHE For1 and GP ACHE Rev1 primers; internal primers (GP ACHE For1 Internal [5′-GACTGCCTCTACCTCAACGT-3′], GP ACHE For2 Internal [5′-GGACCCTTCGCTGAACTACA-3′], GP ACHE Rev1 Internal [5′-ACGTTGAGGTAGAGGCAGTC-3′], and GP ACHE Rev2 Internal [5′-TGTAGTTCAGCGAAGGGTCC-3′]) were designed and synthesized based on results generated from the external primers and were used to acquire the sequence of the entire coding region of the gene. DNA sequence data were generated using an Applied Biosystems 310 Genetic Analyzer (Foster City, CA) and analyzed using Geneious Pro Software (Version 4.0.4, Biomatters Ltd., Auckland, New Zealand). Nucleotide and deduced amino acid sequences of guinea pig AChE were then aligned with and compared to those of human AChE. A structural model of the guinea pig AChE protein was generated using the crystal structure of HuAChE (PDB: 1B41) as a template. The SWISS-MODEL server was used to generate and energy minimize the model [6–8].

2.2. AChE sample preparation

Human red cell ghosts were prepared as described [9] using the modifications suggested by Worek et al. [10]. This approach has been shown to work well with both human red blood cell membranes and those of other mammalian species. Guinea pig red cell ghosts have very low AChE activity (as assessed by hydrolysis of acetylthiocholine) compared to human samples, making precision in reactivation studies difficult to achieve. Briefly, whole blood samples, stabilized with lithium heparin, were obtained from commercial sources (human male whole blood from Bioreclamation, Inc, Hicksville, NY; male guinea pig whole blood from BioChemed Services, Wincheser, VA) and upon their arrival were centrifuged at 4 °C for 20 min at 3000 rpm in a clinical centrifuge. Packed cells were

```
   1 CTGCTCTGGC AGCCATGAGG CCCTGGTGGT GTCCCCAGCA CAAGACCTCC CTGGCCTCCC
  61 CACTCCTCTT CCTCTTCTCC CTCCTGGGAG GAGGAGTGGG GGCTGAGGGC CGGGAAGACC
 121 CAGAGCTGCT GGTGACAGTT CAAGGGGGCC GGCTGCGGGG CATCCGCCTA AAGGCTCCTG
 181 GTGGCCTTGT CTCCGCTTTT CTGGGCATCC CCTTTGCAGA GCCACCTGTG GGCCCCCGAC
 241 GCTTTCTGCC ACCAGAGCCC AAGCGGCCCT GGTCAGGGGT GCTAGATGCT ACAACCTTCC
 301 CTAGTGTCTG CTACCAATAT GTGGACACCT TGTACCCCGG TTTTGAGGGC ATCGAGATGT
 361 GGAACCCTAA CCGTGAGCTG AGTGAGGACT GCCTCTACCT CAACGTATGG ACACCATACC
 421 CTCGGCCTGC ATCCCCTCTC CCTGTCCTTG TCTGGATCTA TGGGGGTGGC TTCTACAGCG
 481 GGGCCTCCTC CCTGGATGTG TATGATGGCC GCTTCCTGAC ACAGGCAGAG AGGATAGTGT
 541 TGGTGTCTAT GAACTACCGG GTGGGAACCT TTGGCTTCTT GGCTCTGCCA GGGAGTCGAG
 601 AGGCACCAGG CAATGTGGGT CTGCTGGATC AGAGGCTGGC ACTGCAGTGG GTGCAAGAGA
 661 ATGTGGCAGC CTTTGGAGGG GACCCCACAT CAGTGACTCT ATTTGGGGAG AGCGCAGGTG
 721 CAGCCTCTGT GGGTATGCAC GTACTGTCCC CCCTCAGCCG GAGCCTCTTC CACAGGGTTG
 781 TGCTGCAGAG CGGCGCACCC AATGGGCCCT GGGCCACAGT AGGCATGGGA GAGGCCCGCC
 841 GCAGGGCCAC TCTGCTAGCC CGTCTTGTAG GCTGTCCCCC AGGCGGCGCT GGAGGCAATG
 901 ACACAGAATT GGTAGCCTGC CTGCGAACAC GGCCAGCTCA GGACTTGGTG GACCACGAGT
 961 GGCATGTGCT GCCCCAGGAA AGCATCTTCC GGTTTTCCTT TGTGCCTGTT GTGGATGGAG
1021 ACTTCCTTAG TGACACGCCT GAGGCCCTCA TCAATGCTGG AGATTTCCAC GGCTTGCAGG
1081 TGCTGGTGGG TGTGGTGAAG GATGAGGGGT CCTATTTTCT GGTTTACGGG GCCCCAGGCT
1141 TCAGCAAAGA CAACGAATCT CTCATCAGCC GGGCCCAGTT CCTGGCTGGA GTACGCATCG
1201 GAGTCCCCCA GGTGAGCGAC CTGGCGGCTG AGGCTGTGGT CCTGCATTAC ACGGACTGGC
1261 TGCATCCTGA GGACCCAGCA CGCTTAAGGG ATGCCATGAG TGCTGTGGTG GGCGACCACA
1321 ATGTCGTGTG CCCAGTAGCC CAGCTGGCTG GGCGACTAGC TGCCCAGGGC GCCCGAGTTT
1381 ATGCCTATGT CTTTGAACAC CGTGCCTCCA CGATCACCTG GCCCCTCTGG ATGGGGGTGC
1441 CCCACGGCTA TGAGATTGAG TTCATCTTTG GACTCCCCCT GGACCCTTCG CTGAACTACA
1501 CCATGGAGGA GAAAATCTTT GCCCAGCGGA TGATGAGATA CTGGGCCAAC TTTGCCCGCA
1561 CAGGGGACCC CAATGACCCG CGCGACGCCA GGGCCCCTCA GTGGCCCGCA TACACGACAG
1621 GAGCGCAGCA GTACGTGAGC CTCAACCTGC GGCCGCTGGA GGTGCGGAGG GGACTGCGCG
1681 CCCAGGCATG CGCCTTCTGG AACCGCTTCC TGCCCAAATT GCTCAGTGCC ACCGACACGC
1741 TGGACGAGGC GGAGCGCCAG TGGAAGGCTG AGTTCCACCG CTGGAGCTCC TACATGGTGC
1801 ACTGGAAGAA CCAGTTCGAC CATTACAGCA AGCAGGACCG CTGCTCAGAC CTGTGACCCC
1861 GGCGGGACCC CACGTCCTCA GGCCCCGCCC GCAGCTGTAT ATACTATTTA TTGAAGGGCA
1921 GGGATATAAC AGACGAGTCC CAGACC
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Fig. 1. The nucleotide sequence of guinea pig *AChE* determined as described in Section 2.1. Positions of primers GP ACHE For1 and GP ACHE Rev1 are underlined, with start and stop codons italicized, bolded, and underlined. The codons corresponding to the putative catalytic triad are indicated in bold italics.

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Hu AChE    1 MRPPQCLLHTPSLASPLLLLLLWLLGGGVGAEGREDAELLVTVRGGRLRGIRLKTPGGPV
GP AChE    1 ...WW.PQ.KT.......F.FS-.............P......Q..........A...L.

Hu AChE   61 SAFLGIPFAEPPMGPRRFLPPEPKQPWSGVVDATTFQSVCYQYVDTLYPGFEGTEMWNPN
GP AChE   60 ............V...........R.....L.....P................I......

Hu AChE  121 RELSEDCLYLNVWTPYPRPTSPTPVLVWIYGGGFYSGASSLDVYDGRFLVQAERTVLVSM
GP AChE  120 ...................A..L.........................T....I.....

Hu AChE  181 NYRVGAFGFLALPGSREAPGNVGLLDQRLALQWVQENVAAFGGDPTSVTLFGESAGAASV
GP AChE  180 .....T.....................................................

Hu AChE  241 GMHLLSPPSRGLFHRAVLQSGAPNGPWATVGMGEARRRATQLAHLVGCPPGGTGGNDTEL
GP AChE  240 ...V...L..S....V.......................L..R........A.......

Hu AChE  301 VACLRTRPAQVLVNHEWHVLPQESVFRFSFVPVVDGDFLSDTPEALINAGDFHGLQVLVG
GP AChE  300 ..........D..D..........I.................................

Hu AChE  361 VVKDEGSYFLVYGAPGFSKDNESLISRAEFLAGVRVGVPQVSDLAAEAVVLHYTDWLHPE
GP AChE  360 ............................Q......I........................

Hu AChE  421 DPARLREALSDVVGDHNVVCPVAQLAGRLAAQGARVYAYVFEHRASTLSWPLWMGVPHGY
GP AChE  420 ......D.M.A.....................................IT...........

Hu AChE  481 EIEFIFGIPLDPSRNYTAEEKIFAQRLMRYWANFARTGDPNEPRDPKAPQWPPYTAGAQQ
GP AChE  480 .......L.....L...M........M..............D...AR.....A..T....

Hu AChE  541 YVSLDLRPLEVRRGLRAQACAFWNRFLPKLLSATDTLDEAERQWKAEFHRWSSYMVHWKN
GP AChE  540 ....N.....................................................

Hu AChE  601 QFDHYSKQDRCSDL*
GP AChE  600 ..............*
```

Fig. 2. Alignment of the deduced amino acid sequences of human and guinea pig AChE. Dots indicate identical amino acids, while dashes represent gaps which have been introduced to aid in alignment. Stop codons are indicated by asterisks.

resuspended in isotonic PBS, and washed twice before final suspension in 6.7 μM phosphate buffer, pH 7.4. Samples were mixed thoroughly and stored at 4 °C overnight to promote hemolysis. The following day the suspension was centrifuged at 20,000 rpm for 30 min, resuspended, and washed twice more before being suspended in 0.1 mM phosphate buffer, pH 7.4 to approximately the same volume at which they were supplied. Red blood cell ghosts were stored at 4 °C until use.

2.3. Acetylcholinesterase inhibition

650 μL of red blood cell ghost suspension was pipetted into a 1.5 mL centrifuge tube and an aliquot of OP nerve agent (tabun (GA), sarin (GB), soman (GD), cyclosarin (GF), O-ethyl-S-(2-diisopropylaminoethyl) methylphosphonothiolate (VX) or O-isobutyl S-(2-diethylaminoethyl)methylphosphonothiolate (VR), obtained from the U.S. Army Edgewood Chemical Biological Center (ECBC, Aberdeen Proving Ground, MD) and found by nuclear magnetic resonance spectroscopy to be ≥97% pure) estimated to be sufficient to inhibit the measured acetylcholinesterase [11] activity was added. After 5 min, a 10 μL sample was withdrawn for measurement of residual activity. If activity remained, the amount of agent needed to inhibit the remaining activity was calculated based on the fraction of activity inhibited by the initial agent addition, and that amount was added. Again, a 10 μL sample was withdrawn for activity measurement. If there was no residual activity, a small (20–50 μL) aliquot of the suspension of red blood cell ghosts was added and again the activity checked. This procedure was repeated until, 10 min after the last addition, there was a barely perceptible (<5% of initial activity) indication of excess active enzyme. It is very important to avoid traces of excess agent in the inhibited enzyme preparations, as they can affect the results of reactivation measurements. Oximes sufficient to provide a final concentration of 1 mM, 300 μM, 100 μM, or 30 μM were added to inhibited samples and aliquots of these mixtures were analyzed for AChE activity at time points 0.25, 2, 5, 10, 15 and 30 min post oxime addition using a standard Ellman assay [11]. For reactions with slow reactivation rates, data at additional time points were collected. For reactivation reactions that proceeded very rapidly, data were continuously collected from a single aliquot after oxime introduction. The extent to which oximes contributed directly to substrate or agent hydrolysis (oximolysis) was determined using control experiments in the absence of enzyme; no significant oximolysis was observed under the conditions used. In all cases, half-times of oxime-mediated reactivation ($t_{1/2}$) were calculated from the resulting data.

3. Results

The determined nucleotide sequence of guinea pig *AChE* (Fig. 1) indicates a gene with a coding region of 1845 base pairs. When aligned with the human *AChE* nucleotide sequence, a homology of 87% identity with 41 gaps was found (data not shown). The deduced amino acid sequence of the unprocessed 614 amino acid residue guinea pig AChE was aligned with that of unprocessed human AChE (Fig. 2). Fifty amino acid differences as well as one deletion were found in guinea pig AChE as compared to human AChE, indicating a 91.7% amino acid sequence identity between the AChE proteins from these two species. The nucleotide and deduced amino acid sequences of guinea pig AChE have been submitted to GenBank and are available under accession #GU453678.

The amino acid sequence of guinea pig AChE was used to generate a structural model based on the crystal structure of human AChE (PDB 1B41) available in the Protein Data Bank (www.pdb.org). The resulting model is displayed in Fig. 3, and indicates that the

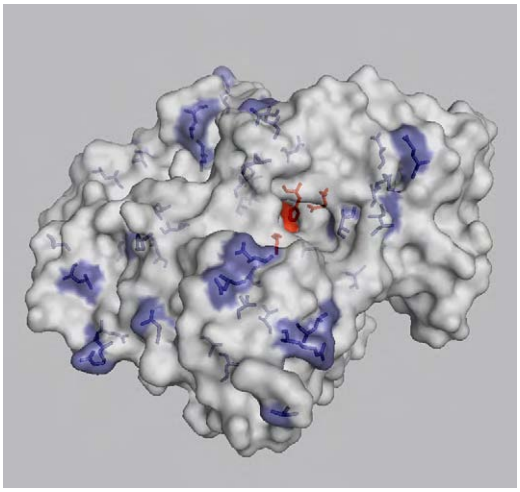

Fig. 3. A structural model of guinea pig AChE based on a crystal structure of human AChE (PDB: 1B41). Amino acid differences between human and guinea pig AChE are highlighted in blue, with those substitutions predicted to be solvent-exposed indicated by blue surface shading. The three amino acids of the putative catalytic active site are shown in red.

amino acid differences between human and guinea pig AChE are distributed throughout the structure of the enzyme, including both solvent-exposed and buried amino acids. None of the residues that differ between human and guinea pig AChE are predicted to be located within the AChE active site pocket, although the relatively conservative substitution of isoleucine for valine at unprocessed protein residue 324 in the guinea pig enzyme (directly to the right of the active site residues in Fig. 3) is predicted to be solvent-exposed and located directly opposite the peripheral binding site [12] at the entrance to the active site gorge.

The reactivation rates were determined for human and guinea pig AChE red blood cell ghosts inhibited by the OP nerve agents GA, GB, GD, GF, VX, or VR, as mediated by the oximes pralidoxime (2-PAM), asoxime (HI-6) or 1,1′-Methylenebis{4-[(hydroxyimino)methyl]pyridinium) dichloride (MMB-4). In most cases one of three concentrations of oxime (30, 100, and 300 μM) was used to determine the half-times of reactivation and normalized first order rate constants shown in Table 1. In those cases where minimal reactivation was detected at lower oxime concentrations, 1 mM oxime was used. Conversely, very rapid reactions provided usable data only at low oxime concentrations.

Consistent with previously reported results [13], human AChE is rapidly reactivated by HI-6 and MMB-4 after inhibition by GB, GF, VX, and VR, with 2-PAM functioning much less efficiently. Reactivation of GA-inhibited human AChE was much slower with all oximes tested, and no reactivation was detectable after inhibition with GD (see Table 1). In contrast, guinea pig AChE was substantially more resistant to oxime-mediated reactivation. The only rates that were comparable between guinea pig and human AChE were found with 2-PAM after inhibition with GB and VR, although a three-fold higher concentration of 2-PAM was needed to reactive guinea pig AChE relative to human AChE. For all of the oximes tested, the reactivation of guinea pig and human AChE after inhibition by G agents was much slower than reactivation of the same enzymes after inhibition by VX or VR.

4. Discussion

The nucleotide and deduced amino acid sequences of guinea pig AChE indicate that guinea pig and human AChE share a high degree of homology, where the residues constituting the catalytic triad are conserved. The only gap in the guinea pig AChE amino acid sequence relative to human occurs at residue 23, near or within the signal sequence of the protein.

Despite the 91.7% homology between the amino acid sequences of human and guinea pig AChE, these enzymes have very distinct oxime-mediated reactivation profiles. Examination of the molecular model of guinea pig AChE (presented in Fig. 3) reveals that while most of the amino acid residues in and near the AChE active site are conserved, guinea pig AChE has an isoleucine at residue 324 in place of the valine found in human AChE. This residue is predicted to be located at the entrance of the active site gorge, directly opposite the peripheral binding site. The increased bulk of an isoleucine residue at this location may hinder the capacity of oximes to either bind or correctly orient within the active site to promote reactivation after nerve agent inhibition. This possibility is supported by the finding here that 2-PAM, a monopyridinium oxime which does not depend on binding to the peripheral site to mediate reactivation, is roughly equivalently effective at reactivating human and guinea pig AChE after inhibition by GB, VX, and VR. Alternatively, the differential capacity of oximes to reactivate AChE from the two species may be a consequence of other amino acid substitutions distal from the active site, or due to differences in the composition of the glycan residues used to post-translationally modify human and guinea pig versions of AChE. The identification of the nucleotide and deduced amino acid sequences of guinea pig AChE provides an opportunity for recombinant expression and characterization of a variant version of guinea pig AChE in which the residue at 324 is replaced with valine, which may help to resolve this issue.

The nerve agent and oxime reactivation experiments indicate that all of the OPs tested efficiently inhibit guinea pig AChE, but that none of the oximes tested (with the exception of MMB-4 used with VR-inhibited enzyme) promote reactivation in a therapeuti-

Table 1
Half-times ($t_{1/2}$) and normalized first order rate constants (K_r) for oxime reactivation of nerve agent inhibited AChE.

Agent	Half-times in minutes of oxime-mediated reactivation (normalized rate constants in M^{-1} min^{-1})					
	Human AChE			Guinea Pig AChE		
	2-PAM	HI-6	MMB-4	2-PAM	HI-6	MMB-4
GA	62 (1.1×10^2)	>7.2×10^3 (<0.96)	85 (82)	>7.2×10^3 (<0.96)	N.D.	N.D.
GB	10 (6.9×10^2)	0.30 (2.3×10^4)	0.78 (8.9×10^3)	10[a] (2.3×10^2)	N.D.[a]	10[a] (2.3×10^2)
GD	N.D.[b]	N.D.[b]	N.D.[b]	N.D.[b]	N.D.[b]	N.D.[b]
GF	>80 (<87)	1.0 (6.9×10^3)	1.1 (6.3×10^3)	N.D.	70 (99)	22 (3.2×10^2)
VX	7.0 (9.9×10^2)	1.8 (3.9×10^3)	3.0 (2.3×10^3)	13 (5.3×10^2)	66 (1.1×10^2)	13 (5.3×10^2)
VR	45 (1.5×10^2)	3[c] (7.7×10^3)	0.80 (8.7×10^3)	43 (1.6×10^2)	27 (2.6×10^2)	2.5 (2.8×10^3)

Rates are indicated in parentheses (K_r = [(ln 2)/$t_{1/2}$]/[Oxime]). All $t_{1/2}$ values were determined using oxime at 100 μM unless otherwise indicated; N.D. = no reactivation detected.

[a] [Oxime] = 300 μM.
[b] [Oxime] = 1 mM.
[c] [HI-6] = 30 μM.

cally relevant timeframe. The inability of any of the tested oximes to reactivate human AChE after inhibition by GD is attributed to the rapid aging of GD that has been documented in human AChE [13]; the results with GD and guinea pig AChE are consistent with similarly rapid aging, although this result might also reflect the generally poor reactivation of guinea pig AChE by any of the oximes tested. In the future, production of purified, recombinant samples of human and guinea pig AChE will allow a direct comparison of the kinetic and thermodynamic parameters of the interactions of OP nerve agents and oximes with these proteins. Such recombinant enzymes will also be of value in determining the extent of stereospecificity displayed by guinea pig AChE as compared to human AChE in the binding of GD and other OP nerve agents. Taken together, the oxime-mediated reactivation results presented here indicate that while guinea pigs may be a useful animal model to assess the *in vivo* safety of oximes, they are not a good model to predict the therapeutic efficacy of oximes in humans.

Conflict of interest

None.

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